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Evaluation of Staining on Rat Brain Tissue with the Dual Combination of Roselle (*Hibiscus Sabdariffa* L.) and Alkanet (*Alkanna Tinctoria* (L.) Tausch) Extracts

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Abstract

Natural colorants are used in many different areas such as textiles, cosmetics, leather, and medicine. The purpose of this study is to identify a natural colorant combination that can be an alternative to Hematoxylin-eosin dye using plant sources. For this, roselle (*Hibiscus sabdariffa*) and alkanet (*Alkanna tinctoria*) plants were used. The Hematoxylin-Eosin (H&E) staining procedure, most used for staining steps, was taken as standard. In this procedure, extracts of *Hibiscus sabdariffa* (Hs) in distilled water instead of eosin, and *Alkanna tinctoria* (At) in ethyl alcohol instead of hematoxylin were used. Seven different experimental sets were created by changing the position of the dyes in the H&E procedure and adding metal salts K Al $(SO_4)_2$ .12 H_2O, $CuSO_4$ .5H_2O. One healthy rat brain tissue preparate was immersed in each of the seven different experimental sets. Another preparate of the same brain tissue was stained with H&E stain for positive control purposes. Preparate not staining was used as negative control. After the staining, images were taken in the light microscope of the tissues of eight preparates which were closed with the entellan. It was observed that the rat brain tissue nucleus and cytoplasm were stained in different colors, especially in the step using copper sulfate and alum. Because plants using in staining have different chemical structures like terpenoids, flavonoids, phenolics.
Keywords: *Alkanna tinctoria;* Hematoxylin-eosin; Natural dyes; Rat brain tissue; Flavonoids.

1. Introduction

For a long time, it has been known that eco-friendly, non-toxic natural dyes have been obtained from three different sources, plants, animals like cochineal, and minerals [1-4]. In the textile and medical sectors, mostly plant full sources are used because they are abundant in nature and contain colorful organic structures [5-7]. Organic structures known as Saponin, Phenol, Tanin, Terpenoid, Flavonoid, and Glikozit are phytochemicals in the structure of plants [8, 9]. Flavonoids, phenols, and tannins are used as coloring organic substances, because of having

chromophore groups. Red, blue, and purple dyes are easily obtained with plants containing flavonoids. Although be using bio or chemical mordants. The dye in brownish color is obtained from plants containing tannin [10]. Since it is important to know the presence and amount of phytochemicals in plants, there are qualitative and quantitative analysis studies related to this. Since the phytochemicals, flavonoids, and phenols are used in fabric, print, fiber, food, medicine, cosmetics, and cell staining, their quantitative content in the plant is also important for dyeing efficiency [11-16]. Roselle, basil, red cabbage, black carrot and alkanet contain high levels of flavonoid and phenolic compounds [17-22]. Additionally, there are many studies on the effects of extracts prepared from various plants such as antifungal, antibacterial, antioxidant, and enzyme activity [23-30].

The alkanet (*Alkanna tinctoria*) plant, which is one of the plants used in this study, contains the naphthoquinone group dyestuff [31]. Biological studies have been conducted on the wound healing properties of Shikonin and Alkanni, the enantiomeric forms of naphthoquinone [32]. There are also studies conducted on the biological activities of pigments contained in the alkanet [33-35]. In a study by Alwahibi and Perveen [36], it was determined by GC-MS analysis that the ethanol extract obtained from the roots of the alkanet plant contains more active compounds than water, which confirmed that the extract obtained with ethanol had more antibacterial effects.

The roselle (*Hibiscus sabdariffa*), which belongs to the family of Malvaceae, is a 2.0-2.5m tall plant [37]. There are studies about the contained phytochemicals and organic acids in the extracts of the plant in water, methyl alcohol, and other solvents [38-42]. Dark red ones are reported to have high phenolic content [43]. Therefore, there are some studies of these extracts using strong antibacterial, antioxidant, antimicrobial, pharmacological, and medical purposes and even in the treatment of hypertension [44-48]. Milena et al stated that the extracts obtained from the hot and cold water of the roselle plant varied especially in terms of phytochemical content and that there were more phenolic and flavonoid substances in the extract obtained by the heated treatment [49, 50]. In this study, dark staining was performed in the cytoplasm with the extract obtained in the heated treatment. The chelate structure formed between proteins found in the cytoplasm and the phenolic compounds in the roselle extract in the presence of metal salt is shown in Figure1.

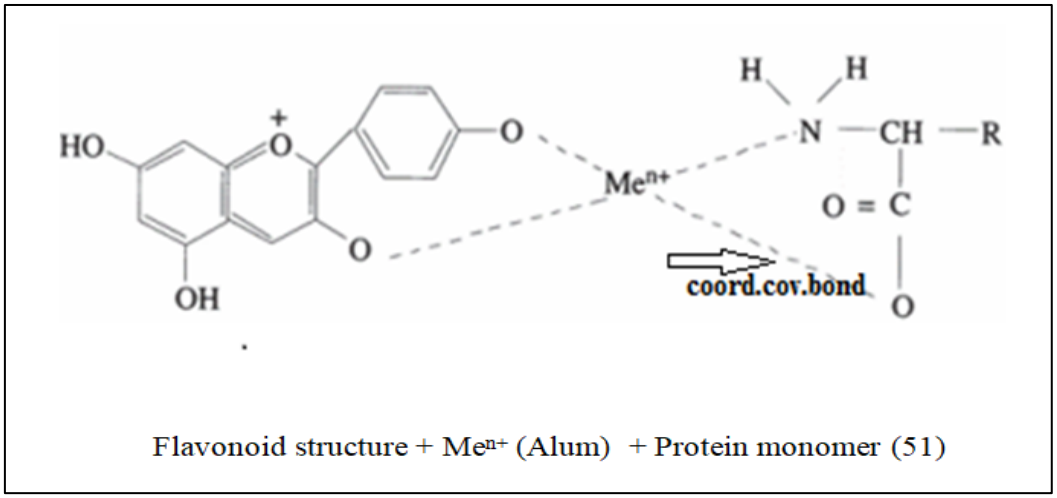

Figure-1. Chelate complex structure occurred between protein and dyestuff with a metal salt [51]

Flavonoids exhibit antioxidant properties in our metabolism by chelating with transition metals, mainly Fe (II), Fe (III), and Cu (II), which are involved in free radical-generating reactions. Metal-flavonoid chelates are stronger free radical scavengers than alone flavonoids. They have an important role in protecting from oxidative stress. Extensive Physico-chemical studies have been conducted to reveal the chemical structure, chelation sites, the effect of the metal/ligand ratio on the structure of the complexes, the ability of flavonoids to bind metal ions to reveal the origin of their powerful biological effects [52].

Chelate molecule was formed between the flavonoid structure and metal-protein monomer by adding metal salts as mordants. However, there have been studies on the use of other biomordants in recent years [53]. Biomordants can be also used between different plant molecules [54]. In addition to the use of different sources in the procurement of mordant the same method was followed in the dyestuff sources and used various types of algal as a dye source [55]. Specially, biomordants and antibacterial palnt extracts mostly use in textile industry to dye fibres. There are a alot of studies about this [56-58].

2. Material and Methods

Hibiscus sabdariffa and *Alkanna tinctoria* were obtained from local markets of Kayseri province. All solvents were of reagent grade without any further purification. All purchased chemicals such as xylene; alcohol, chloroform, and ammonia were obtained from Merck (Darmstadt, Germany).

2.1. Animals and Experimental Protocol

In this study, to avoid new animal deaths, the brain tissues (in the control group) remaining from a previous study were used. The protocol used in this study was endorsed by the Erciyes University Animal Experiments Local Ethics Committee (Protocol No: 14/79). Sexually mature female Wistar inbred albino rats were obtained from the Experimental and Clinical Research Center of Erciyes University (Kayseri, Turkey). Wistar albino rats were delivered spontaneously and after 10 days they were included in the study. The rats were fed with pellet food and had free access to water in a room, they have been kept the constant temperature at 23 °C in a 12-hour day and night cycle.

2.2. Preparation of the Tissue Preparates

At the end of the study, the rats were sacrificed under general anesthesia by an intraperitoneal injection of ketamine hydrochloride (75 mg/kg, Ketalar) + xylazine hydrochloride (10 mg/kg), and brain tissue samples were obtained. Brain tissues of the rats were fixed for 24 hr in 10% neutral formalin for histopathological analysis. These samples were then kept in the graded ethanol series and embedded in paraffin. Paraffin blocks were cut into 5 µm segments using a microtome. The 5 µm cross-sections taken from the tissue paraffin blocks for staining were then put through the staining procedure to see the effect of the *Hibiscus sabdariffa* and *Alkanna tinctoria*. In this study. we most used semiqualitative scoring system. The color intensity of staining was evaluated as negative (−), weak (+), medium (++), and very intense (+++).We used Qualitative scoring with weak (+), medium-strong (++), very intense (+++, dark) concepts according to the color darkness and lightness in the nucleus and cytoplasm of the stained cells. We did not use quantitative values such as the number of stained nuclei, markers and total cell number in the evaluation [59-61].

2.3. Deparaffinization of Tissue

Paraffin was removed from the preparates by the classical method. Preparates were left in an oven at 40ºC for one night. The next day, they were removed from the oven and allowed to cool to room temperature for 3-5 min [62].

2.4. Preparation of Hibiscus Sabdariffa Extract

The dried *Hibiscus sabdariffa* was ground to a dark red-black powder using manual grinding machines (Waring, commercial). The dried powder of the plant weighed 50 g (Shimadzu bl 3200). The powder was boiled in a beaker containing 100mL distilled water (1g: 2mL) in a beaker for thirty minutes. When cooled, the 50 % (w/v) extract was filtered two times with Whatman No.1 filter paper. To kept stability, and to prevent degradation of the filtrate was stored in the refrigerator at 4°C, until staining processes.

2.5. Preparation of Alkanna Tinctoria Extract

Alkanna tinctoria was ground to a dark black-blue powder using manual grinding machines (Waring, commercial). The dried powder of the plant weighed 50 g (Shimadzu bl 3200). The powder was waited in a closed beaker containing 100mL % ethanol (1g powder:2mL solvent) for 24 hours at room temperature at dark place. Then the extract was filtered two times with Whatman No.1 filter paper. The filtrate was heated until half of the ethanol had evaporated. The remaining blue-purple colored filtrate was used as the dye solution. To kept stability, and to prevent degradation of solution the filtrate was stored in the refrigerator at 4°C, until staining processes.

2.6. Phytochemical Analysis of Extracts

The phytochemical analysis steps below were applied separately to the extract of *Alkanna tinctoria* obtained in ethyl alcohol and the aqueous extract of *Hibiscus sabdariffa* [63].

2.7. Test for Saponin

2 ml extract was placed in a test tube and shaken vigorously. The formation of stable foam was taken as an indication of the presence of saponin.

2.8. Test for Phenol and Tannin

2 ml extract was mixed with 2 ml of 2 % solution of ferric chloride ($FeCl_3$). A blue-green or black coloration indicated the presence of phenol and tannin.

2.9. Test for Terpenoid (Salkowski's Test)

2 ml extract was mixed with 2 ml of chloroform. Then 2 ml of concentrated sulfuric acid was added carefully and shaken gently. A reddish-brown coloration of the interphase was formed to show positive results for the presence of terpenoid.

2.10. Test for Flavonoid (Zinc-Hydrochloride Reduction Test)

2 ml extract was mixed with zinc dust and concentrated hydrochloric acid was added dropwise. It gave a red color after a few minutes indicating the presence of flavonoid.

2.11. Test for Glycoside

2 ml extract was mixed with 2 ml of glacial acetic acid containing 2 drops of 2 % $FeCl_3$. The mixture was poured into another tube containing 2 ml of concentrated sulfuric acid. A brown ring in the interphase indicated the presence of glycoside [63].

2.12. Positive Control Staining

The *Hematoxylin-Eosin* staining process was applied to the deparaffinized preparates as below.

Xylene 3 min, Xylene 3 min, Xylene 3 min, Xylene 3 min, Alcohol 3 min, Alcohol 3 min, Alcohol 3 min, Alcohol 3 min, Washed 3 min, Hematoxylin (H) 6 min, Washed 3 min, 1 % Ammonia water 2 min, Washed 3 min, Eosin (E) 10 min, Washed 3 min, Alcohol 3 min, Alcohol 3 min, Alcohol 3 min, Alcohol 3 min, Xylene 3 min, Xylene 3 min, Xylene 3 min, Xylene 3 min, and Closed with entellan [64].

Photographs of the tissues on the slides were taken at 100× magnification by light microscopy (Olympus BX-51, Japan).

2.13. Natural Staining Method:

Hibiscus sabdariffa alum and *Alkanna tinctoria* with/ without alum- $CuSO_4$ $5H_2O$ extract instead of hematoxylin-eosin was used in the Hematoxylin-eosin routine staining process. Paraffin-free tissue preparations were dipped into the following solutions for the given time and order. Seven rat brain tissue preparates were separately used in the seven trial experimental sets given below.

2.13.1. Set 1

Xylene 3 min, Xylene 3 min, Xylene 3 min, Xylene 3 min, Alcohol 3 min, Alcohol 3 min, Alcohol 3 min, Alcohol 3 min, *Alkanna tinctoria* (At) ethanol extract +3g alum 20 min, Washed 3 min, Dried 10 min, Xylene 3 min, Xylene 3 min, Xylene 3 min, and Mounted with entellan.

2.13.2. Set 2

Xylene 3 min, Xylene 3 min, Xylene 3 min, Xylene 3 min, Alcohol 3 min, Alcohol 3 min, Alcohol 3 min, Alcohol 3 min, *Hibiscus sabdariffa* (Hs) water extract +5g alum 20 min, Washed 3 min, Dried 10 min, Xylene 3 min, Xylene 3 min, Xylene 3 min, and Mounted with entellan.

2.13.3. Set 3

Xylene 3 min, Xylene 3 min, Xylene 3 min, Xylene 3 min, Alcohol 3 min, Alcohol 3 min, Alcohol 3 min, Alcohol 3 min, *Alkanna tinctoria* (At) ethanol extract + 3g alum 20 min, 1% Acid alcohol 2min, 1% Ammonia water 2 min, *Hibiscus sabdariffa* (Hs) water extract + 5g alum 20 min, Washed 3 min, Alcohol 3 min, Alcohol 3 min, Alcohol 3 min, Alcohol 3 min, Xylene 3 min, Xylene 3 min, Xylene 3 min, and Mounted with entellan.

2.13.4. Set 4

Xylene 3 min, Xylene 3 min, Xylene 3 min, Xylene 3 min, Alcohol 3 min, Alcohol 3 min, Alcohol 3 min, Alcohol 3 min, *Hibiscus sabdariffa* (Hs) water extract + 5g alum 20 min, 1% Acid alcohol 2min, 1 % Ammonia water 2 min, *Alkanna tinctoria* (At) ethanol extract +3g alum+2g $CuSO_4$ $5H_2O$ 20 min, Washed 3 min, Alcohol 3 min, Alcohol 3 min, Alcohol 3 min, Alcohol 3 min, Xylene 3 min, Xylene 3 min, Xylene 3 min, and Mounted with entellan.

2.13.5. Set 5

Xylene 3 min, Xylene 3 min, Xylene 3 min, Xylene 3 min, Alcohol 3 min, Alcohol 3 min, Alcohol 3 min, Alcohol 3 min, *Hibiscus sabdariffa* (Hs) water extract 20 min, 1% Acid alcohol 2min, 1% Ammonia water 2 min, *Alkanna tinctoria* (At) ethanol extract 20 min, Washed 3 min, Alcohol 3 min, Alcohol 3 min, Alcohol 3 min, Alcohol 3 min, Xylene 3 min, Xylene 3 min, Xylene 3 min, and Mounted with entellan.

2.13.6. Set 6

Xylene 3 min, Xylene 3 min, Xylene 3 min, Xylene 3 min, Alcohol 3 min, Alcohol 3 min, Alcohol 3 min, Alcohol 3 min, *Alkanna tinctoria* (At) ethanol extract 20 min, Washed 3 min, Dried 10 min, Xylene 3 min, Xylene 3 min, Xylene 3 min, and Mounted with entellan.

2.13.7. Set 7

Xylene 3 min, Xylene 3 min, Xylene 3 min, Xylene 3 min, Alcohol 3 min, Alcohol 3 min, Alcohol 3 min, Alcohol 3 min, *Hibiscus sabdariffa* (Hs) water extract 20 min, Washed 3 min, Dried 10 min, Xylene 3 min, Xylene 3 min, Xylene 3 min, and Mounted with entellan.

Photographs of the tissues on the slides after the five dyeing processes were taken separately at 100× magnification by light microscopy (Olympus BX-51, Japan).

3. Results

Analysis was done separately to detect the phytochemical content of the alkanet (*Alkanna tinctoria*) and roselle (*Hibiscus sabdariffa)* extracts. Analysis results are given in Table 1. Since *Hibiscus sabdariffa* plant contains flavonoid groups intensely like cyanidin, delphinidin. The result of the flavonoid qualitative analysis is shown as '++ ' in Table 1. Since the alkanet plant contains phenol -tannin intensely, the result of the phytochemical analysis is shown in Table 1 with '++'. The Flavonoid content of alkanet is shown with '_ 'because it is almost non-existent. The saponin content of both plants was given with the '+' sign, as varying amounts of foaming were observed in the extracts.

Table-1. Phytochemical analysis results of plant extracts

Extract	Phenols & tannin	Saponin	Terpenoid	Glycoside	Flavonoid
H. sabdariffa	+	+	-	-	++
Alkanet	++	+	-	-	-

Note: positive (+): the presence of constituent; (++) the most presence: negative (-): the absence of constituent.

As seen Table 1**,** *H. sabdariffa* and *A. tinctoria* have contained different chemical structures like flavonoid and terpenoid. The methods used to prepare extracts from both plants are also different. In preparing of *Hibiscus sabdariffa* extract was used a hot process. Also, preparing alkanet extract used a cold process. So, different organelles in tissue were dyed at different colors.

Table-2. Step by step results of staining tissues

The color intensity of stain on tissue Color of cell elements				
Set Number	**Cytoplasm (cyt)**	**Nucleus (n)**	**Cytoplasm (cyt)**	**Nucleus (n)**
1	+	++	Light blue	Blue
2	+	++	Light pink	Pink
3	+++	+++	Dark blue	Dark blue-purple
4	++	++	Pink-red	Dark purple
5	+++	+++	Dark pink	Dark pink-purple
6	_	_	No color	No color
7	_	_	No color	No color

Note: negative (-): absent coloring, positive (+): weak coloring, (++): medium coloring, (+++): strong coloring

Seven different sets were prepared from extracts after phytochemical analysis. The 1st and 2nd sets were studied without alum as in sets 6 and 7. But since there was no staining, their photographs were not given in this study. In the application without alum, only in the 5 th experimental set, the nucleus and cytoplasm were stained in a dark color, but there was no color difference between the nucleus and cytoplasm. In the other 4 sets, the rat brain tissue taken from the control group was stained using metal salts. Thus, both the nucleus and cytoplasm were stained in darker and different colors. The examination of the light microscopy images of dyed tissues was done. Results, according to the color and the intensity of the staining are given in Table 2.

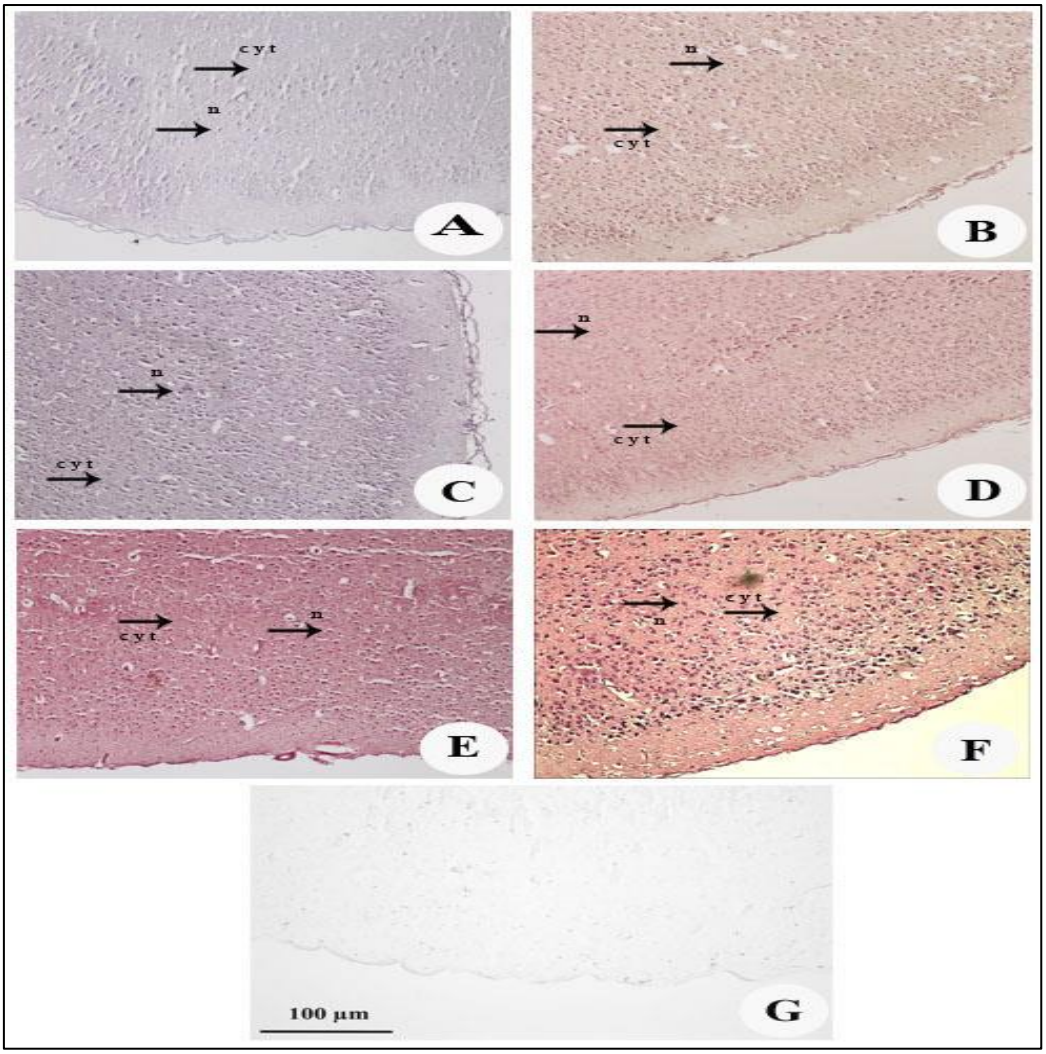

Figure-2. Photographs of staining on rat brain tissues according to different dyeing sets A) Set 1: *Alkanna tinctoria*, B) Set 2: *Hibiscus sabdariffa*, C) Set 3: *Alkanna tinctoria* + *Hibiscus sabdariffa*, D) Set 4: *Hibiscus sabdariffa* + *Alkanna tinctoria*, E) Set 5: *Hibiscus sabdariffa* + *Alkanna tinctoria*, F) Stained with H&E (100×). Photographs of staining on rat brain tissues according to different dyeing sets A) Set 1: *Alkanna tinctoria*, B) Set 2: *Hibiscus sabdariffa*, C) Set 3: *Alkanna tinctoria* + *Hibiscus sabdariffa*, D) Set 4: *Hibiscus sabdariffa* + *Alkanna tinctoria*, E) Set 5: *Hibiscus sabdariffa* + *Alkanna tinctoria*, F) Stained with H&E (100×) (Positive control), G) Negative control slide. (n: nucleus; cyt: cytoplasm)

In the tissue, where the staining process was applied according to Set 1, the cytoplasm and the nucleus were stained in the same blue color, with the effect of the *Alkanna tinctoria* extract. The same situation occurred in pink with *Hibiscus sabdariffa* in set 2. Since the alkanet (*Alkanna tinctoria*) extract was used in set 3. The nucleus and cytoplasm staining in blue color was a darker blue-purple with the effect of the *Hibiscus sabdariffa*. However, since the dual combination dyeing process was used firstly in the dyeing process in set 4, the nucleus and cytoplasm were stained in two different colors in this staining process. In set 5, since *Hibiscus sabdariffa* was used in the first stage, the nucleus **(n)** and cytoplasm **(cyt)** were stained in dark pink. In other words, the color of the dye in which the tissue was first immersed was showed the color of the nucleus and cytoplasm, in Set 3 and 5. However, when the second metal salt was used, like in set 4, the color difference between the nucleus and the cytoplasm was provided. In sets 6 and 7, the extracts were used without metal salt, but positive results could not be obtained. Light microscopy images of the tissues in 5 sets with positive staining results were taken from the central region (cortex medulla) where the nucleus was abundant in rat brain tissue and is shown in Figure 2.

4. Quality and Stability of Natural Dye Extracts

In this study, two types of natural dye solutions were used. The water extract of *Hibiscus sabdariffa* and the extract of Alkanet in ethyl alcohol was used in tissue dyeing. Storage conditions of these extracts, solution pH value, solvent medium are factors affecting the quality of the dye solution. Since hibiscus extract is also added to foods as a colorant, its color quality and stability are important. Therefore, the stability and quality of the dye solution are increased by adding ascorbic acid to the *Hibiscus sabdariffa* extract [65, 66]. The first case for the long-term use of this solution is to add less ascorbic acid or acid buffers to the solution and conserved in refrigerator This situation may cause some lightening of the dye color in the tissue, but this lightening can be eliminated by increasing the dye extract concentration. The second situation is to use the solid dye obtained by removing the water from the aqueous

extract obtained from the hibiscus. This solid dye is dissolved by adding a mixture of ethyl alcohol + water and texture dyeing is done. The use of alcohol as a solvent will increase the quality of the natural dye solution more than water. There are recent improvements about dye-sensitized solar cells to measure the quality, stability of natural dye extracts [67-69]. There are positive studies about the stability of alkanet in water and other solvents, and its antioxidant properties [70]. Besides, the ethanol extract of alkanet has been found to be resistant to S. aureus [71]. Since the alkanet dye extract, we used in this study is prepared only with ethyl alcohol, the quality and stability of the dye solution will also be better.

5. Discussion and Conclusion

In addition to colored organic structures such as flavonoids, there are many different types of fatty acids, esters, alcohols, and ketones in the extracts of plants [72]. However, flavonoids, tannins, and phenolic compounds in an extract play an effective role in staining studies. There are many studies in which dyes taken from colorful plants were used for dyeing different tissue sections taken from helminths, rats, and humans [62]. In a study conducted by Chomeana, *et al.* [73]. The human spermatozoa was dyed with the dye extracts obtained from the black rice (*Oryza Sativa*), blue butterfly ivy (*Clitoria ternatea*) and fresh roselle (*Hibiscus sabdariffa*) by using the cold keeping method. In the comparison made based on dip quick dyes and rapid PAP, it was reported that the extract obtained from black rice has the potential to be used as an alternative dye in the evaluation of sperm morphology [73]. In another study, it was seen that the nucleus and cytoplasm of liver cells from, animal tissues were stained a purple color with extracts from both fresh, and dried black plum fruits and black mulberries. In this study, carmine and hematoxylin-eosin dyes were used for comparison purposes [74, 75]. In a study conducted by Avwioro, *et al.* [76] the powder dyestuff from the ethanol extract of the Curcuma longa plant was obtained. Dye solutions were prepared by separately dissolving the powder dye, in 1% distilled water, 70% ethanol, 1% acetic acid, and alum in ethanol. They used the dye solutions to stain different tissues, blood, and muscles in different, vivid colors. In a 1% aqueous solution of the dye, the yellow cytoplasm color in the cells was described as staining like eosin, and in other dye solution, the blue-black nucleus staining was evaluated as staining like hematoxylin [76].

In a study carried out by Akinloye et al., the extracts of Bixa orellana, Curcuma domestica, Lonchocarpus cyanescens, and Pterocarpus osun plants, were obtained in ethanol solvent by a soxhlet extractor. Then, the solvent was evaporated to obtain dye powder. It was attempted to dye the fiber and vessel parts of woody textures with the dye powder [77]. Extracts of sugar beet, China rose and red rose plants in water and alcohol were used to stain tissue segments of platyhelminths and various parasite species. In particular, the different histological segments of platyhelminths were stained in different colors with three plant extracts in acidic and basic solutions [78].

In other studies, the cytoplasm and nucleus were stained in different colors with dyes in different solvents obtained from a single plant and the staining was performed in different sections, not in a single tissue section. In this study, staining was done to dye the nucleus and cytoplasm in different colors on a single section in the same tissue with the extracts obtained from two different plants. In particular, basic plant extracts have been used to interact with the acidic DNA and RNA structures present in the nucleus. The fatty acids in the basic extract increased the dyeing efficiency in the nucleus by saponification. Thus, the nucleus color was seen as a blue-purple color. The chelate complex occurs between dye molecules in the acidic plant extract with the metal salt and monomer structures of protein in the cytoplasm. Thus, cytoplasm color appeared as pink. As seen in Table 1, there are more water-insoluble naphthoquinone, terpenoid group phenolics in the structure of the alkanet plant, whereas more water-soluble phenolic anthocyanin and acidic structures in the structure of the roselle facilitated double staining in the cell.

Textile materials (silk, wool, cotton) and natural dyes are bio-sourced materials and contain organic molecules in their structures. Therefore, bio-bio interactions can be considered to be effective based on the positive dyeing results of natural dyes on textile materials. But, it is difficult for natural dyes to interact with synthetic polymers and organics. There are mostly molecules in protein structure in the structure of textile materials such as silk and wool. Proteins are the most important and abundantly produced molecules in human and animal metabolism. Considering that natural dyes interact with protein structures in natural textiles, the use of natural dyes in tissue dyeing will also be positive. Based on this hypothesis, metal salts used in the dyeing of natural textile materials were used in staining rat tissues. It was observed that harmless chemicals and metal salts, especially iron and alum mordants, were used in the staining procedure of hematoxylin, which is one of the histological dyes currently used in histology. Therefore, in this study, plant dyes were used instead of natural dye and eosin in the hematoxylin staining procedure, and the staining of vibrant, distinctly colored cell elements was carried out in rat tissues. In future studies, it is necessary to bring innovations in tissue dyeing by applying developments such as bio-mordanting, ozone, microwave technics used in dyeing textile materials.

The use of a more natural, easy-to-obtain *Hibiscus sabdariffa* plant instead of a synthetic dye such as Eosin, and the use of a natural easily available *Alkanna tinctoria* plant instead of hematoxylin are important for the original

value and innovation of the study. The use of *Hibiscus sabdariffa-Alkanna tinctoria* may be suggested instead of Hematoxylin-eosin (H&E), which is widely used in medical laboratories as a nucleus-cytoplasm dye in the same tissue.

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Ethical Approval and consent to participate

Ethical approval to report this case series was obtained from * Erciyes University Animal Experiments Local Ethics Committee, Kayseri, Turkey. All procedures in this study were conducted in accordance with the *Erciyes University Animal Experiments Local Ethics Committee, Kayseri, Turkey (14/79)* approved protocols.

Conflict of interest

The authors declared that there is no conflict of interest.

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